THE MICRO-ELEMENT NUTRITION OF *NOSTOC MUSCORUM*

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The Myxophyceae or blue-green algae are the most primitive or least differentiated group of photosynthetic organisms in the plant kingdom. In common with photosynthetic bacteria, they possess neither chloroplasts nor nuclei, and certain species such as *Nostoc muscorum* also are able to fix atmospheric nitrogen (Allison *et. al.*, 1937; Fogg, 1947; Williams and Burris, 1952; Magee and Burris, 1954). It was pointed out by Williams and Burris (1952) that the ability to fix atmospheric nitrogen should be advantageous in the large scale culture of algae intended for use as food.

The macronutrient requirements of blue-green algae have been investigated notably by Chu (1942), Hecker (1950), Gerloff *et al.* (1950, 1952), Allen (1952), Allen and Arnon (1955), and Kratz and Myers (1955). Chu formulated 17 nutrient media for growing different algae, the No. 10 medium being the one most suitable for blue-green algae and the one used in this investigation. Minimum concentrations of essential major elements for optimum yields were obtained for *Coccochloris peniocystis* and *Microcystis aeruginosa* by Gerloff *et al.* (1950, 1952), and they reported that a pH of 10 is optimal.

The essentiality of molybdenum for nitrogen fixation and for assimilation of fixed inorganic nitrogen by *Nostoc* and *Anabaena* was reported by Bortels (1940). The essentiality of boron for the growth of *Nostoc muscorum* was demonstrated by Eyster (1952), and recently Holm-Hansen *et al.* (1954) have shown that cobalt is essential.

The present investigation was undertaken to establish the micronutrient requirements of the blue-green alga, *Nostoc muscorum*. In addition, information was obtained on the levels of concentration at which these various micronutrients become toxic to its growth.

MATERIALS AND METHODS

The strain of *Nostoc muscorum* which was employed in this study was obtained as a pure culture from Drs. Gerloff and Skoog, University of Wisconsin. Precautions were taken to prevent bacterial contamination and cultures which gave a positive test for bacteria with lactose broth were discarded. Cultures were illuminated continuously with flourescent light of 100 f.c. intensity at a temperature of approximately 30° C.

Nutrient solutions having approximately the same content of macronutrients as Chu 10 medium were prepared with spectroscopically pure compounds obtained from Johnson, Matthey and Co., Ltd. (73–83 Hatton Garden, London, England). Since not all of the compounds used by Chu could be obtained in spectroscopically pure form, a modified formula was employed (table 1). The modified Chu No. 10 medium contained all the macronutrient elements, except nitrogen, at the same concentration as recommended by Chu. The nitrogen supply was doubled. The full nitrogen requirement as specified by Chu was supplied as nitrate and an equal amount of ammonium nitrogen (removed from these alkaline cultures during autoclaving) was also included. Boron, manganese and molybdenum were supplied at a concentration of 0.1 p.p.m. unless otherwise stated.

Chu No. 10 medium was also prepared with c.p. grade chemicals for measurements of growth at high levels of all micronutrients. The microelements, other than

TABLE 1

Composition of culture solution used for low level microelement nutrition experiments with Nostoc muscorum

Specpure compound	Grams per liter	P.p.m. of essential elements
$CaCl_2$	(0.98 ml. 1% Ca)[a]	Ca..... 9.8
NH_4NO_3	0.0390	N.....13.6
KH_2PO_4	0.0079	K..... 2.3
		P..... 1.8
KCl	0.0042	K..... 2.2[b]
$MgSO_4 \cdot 7H_2O$	0.0250	Mg..... 2.5
		S..... 3.2
$NaHCO_3$	0.1000	C.....14.3
$FeSO_4 \cdot 7H_2O$	0.00137	Fe..... 0.28
$MnSO_4 \cdot 7H_2O$	0.000504	Mn..... 0.1
H_3BO_3	0.000580	B..... 0.1
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.000184	Mo..... 0.1

[a]Already in solution.
[b]Additional. Total K is 4.5 p.p.m.

TABLE 2

Growth of Nostoc muscorum *at various low levels of each of the four essential micro-elements*

Micro-element	Concentration	Cell count at end of 4 wk.	6 wk.	8 wk.
	p.p.m.	x10⁶/ml.	x10⁶/ml.	x10⁶/ml.
Iron	0	8		
	0.02	17		
	0.04	15		
	0.08	24		
	0.2	23		
	0.4	22		
	1.2	28		
	1.6	22		
	2.0	26		
Manganese	0	4	11	7
	0.001	16	28	36
	0.01	14	36	42
	0.1	8	30	42
	1	12	26	48
	10	9	16	24
Boron	0	19	22	31
	0.001	19	21	34
	0.01	23	27	38
	0.1	20	30	51
	1	23	31	45
	10	11	20	29
Molybdenum	0			23
	4×10^{-8}			46
	4×10^{-7}			27
	4×10^{-6}			33
	4×10^{-5}			45
	4×10^{-4}			54
	4×10^{-3}			64

iron, found to be essential for *Nostoc* in this study were added to the unmodified Chu No. 10 solution to the extent of 0.1 p.p.m.

The conductivity water which was employed in preparing all solutions and media was distilled and was further purified by two passages through a Crystalab Deeminizer (Crystal Research Laboratories, Inc., Hartford 3, Conn.).

TABLE 3

Growth of Nostoc muscorum *at higher than optimal concentrations of each essential micro-element to determine toxic levels*

Micro-element	Concentration	Cell count at end of 4 wk.	Cell count at end of 8 wk.
	p.p.m.	x10⁶/ml.	x10⁶/ml.
Iron	2	33	40
	4	34	45
	6	34	46
	8	37	44
	10	38	45
	20	34	42
	40	20	30
	60	4	5
	80	3	6
	100	1	1
Manganese	1		48
	10	32	46
	20	29	44
	40	23	26
	60	14	21
	80	5	3
	100	3	8
Boron	0.1	37	44
	1	31	50
	10	28	28
	20	25	24
	40	24	28
	60	23	20
	80	17	14
	100	1	6
Molybdenum	0.4	40	54
	2	42	54
	4	44	56
	10	44	48
	20	42	51
	40	38	46
	60	38	49
	80	32	51
	100	42	49
	200	27	
	300	33	
	400	16	
	500	0.1	

The culture vessels were 250-ml. Pyrex Erlenmeyer flasks closed with cotton plugs except those illustrated ones of the iron series which were covered with clean 50–ml. beakers. The volume of the nutrient solution in each flask was 100 ml. The culture flasks were static except for manual shaking usually once daily. Neither air nor CO_2 was bubbled through the flasks. Boron-free Corning flasks were used to establish the essentiality of boron. All glassware was supercleaned by the process of Waring and Werkman (1942) with the following modification.

Between the treatments with alcoholic KOH and with *aqua regia*, the vessels were subjected to 1 percent sodium versenate which has a strong affinity for metal ions. Metals replace sodium in the versenate molecule and are easily removed due to the high solubility of metal-versenate compounds.

After autoclaving, the cooled nutrient solution in each culture flask was inoculated with a loopful of bacteria-free *Nostoc muscorum*. For clear-cut demonstrations of deficiency symptoms, inoculations were made from a culture already

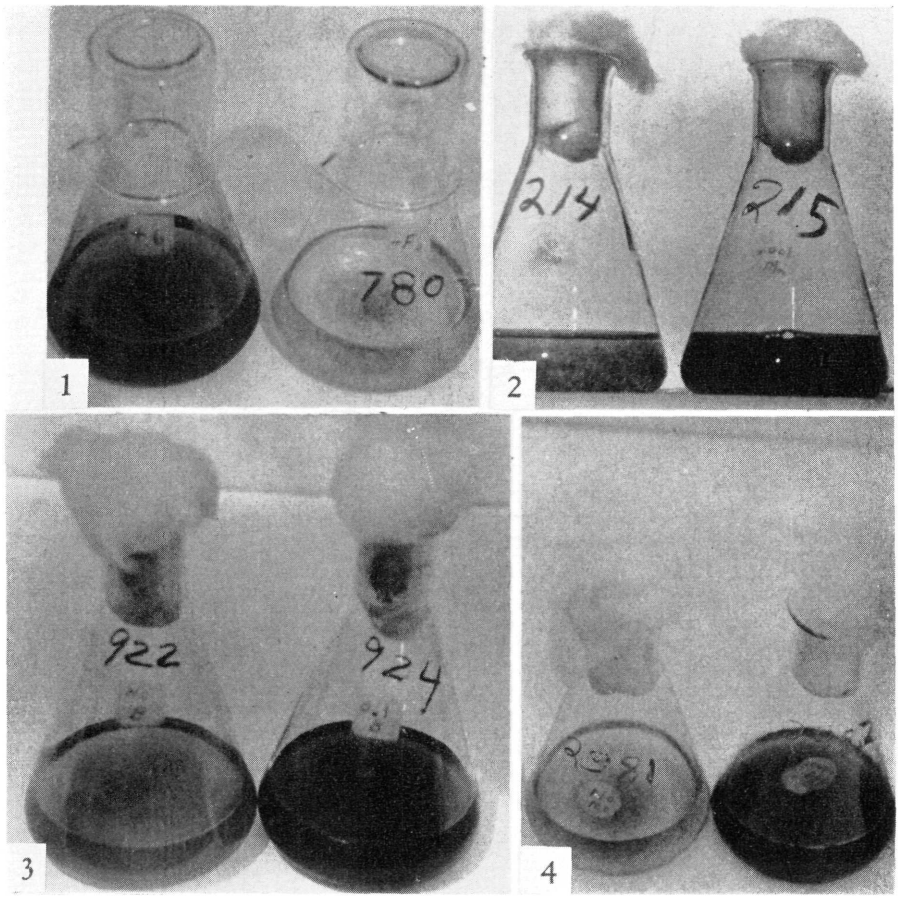

FIGURE 1. Culture on left with iron and culture on right without iron added to the medium.
FIGURE 2. Culture on left without manganese and culture on right with 0.001 p.p.m. manganese added to the medium.
FIGURE 3. Culture on left without boron and culture on right with 0.1 p.p.m. boron added to the medium.
FIGURE 4. Culture on left without molybdenum and culture on right with 0.0004 p.p.m. molybdenum added to the medium.

deficient in the element being studied, and each culture had already been subcultured several times. The boron-deficient series, for example, represented the fifth subculturing from successive two to four-month old boron-deficient cultures. The inoculum cultures distinctly showed the deficiency symptoms and contained no large clumps. It was observed that younger cultures might be somewhat clumped and less homogeneous than older cultures.

Measurements of growth were made by counting cells at the end of 4, 6 and/or 8 weeks. Cultures were brought up to 100-ml. volumes by replacing the evaporated portion with distilled water, and then were poured into a Waring blender to disperse the clumps and homogenize the cultures. Initial blending for two minutes and short blendings before the removal of subsequent samples proved to be adequate for obtaining representative samples. For cell counting a Sedgewick-Rafter algal counting chamber together with a Whipple occular micrometer was used. Five squares, randomly chosen, were counted on each of four samples making a total of twenty counts on each culture. Each sample was required to have a volume of one

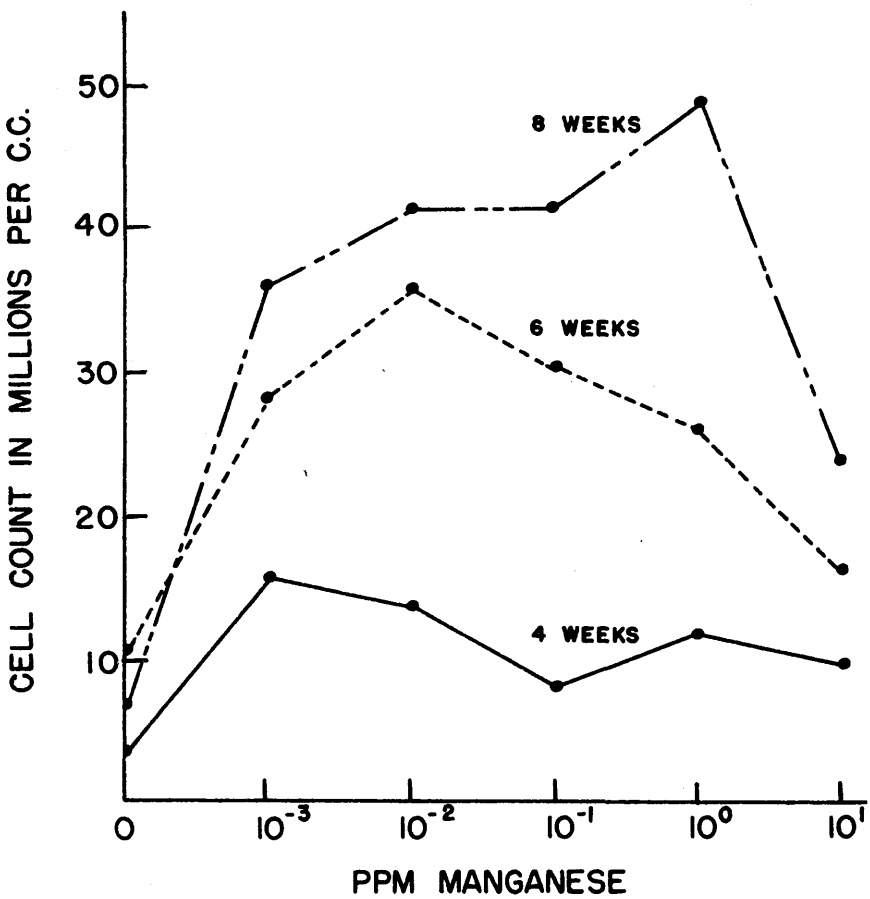

FIGURE 5. Growth of *Nostoc muscorum* at successively higher levels of manganese in the medium.

ml to fill the Sedgewick-Rafter counting chamber. Usually the ml. sample taken directly from the Waring blender was diluted 10 to 20 times, and then a milliliter of this thoroughly mixed dilution was transferred to the counting chamber. Cultures diluted ten times gave direct readings of millions of cells per ml. of the original culture.

RESULTS

It was found that four micronutrient elements are essential to the growth of *Nostoc muscorum*. These elements are iron, manganese, boron and molybdenum. Table 2 presents data on the growth of the alga at various low levels of each of these

elements. At "0" level there was still some growth, but it is assumed that with absolute freedom from the essential micronutrient element there would have been no growth whatsoever. Possible sources of these elements are sub-spectroscopic contamination of the macronutrient "specpure" salts, glassware even though super-cleaned, and the inoculum. It has been shown by Meagher *et al.* (1952) that "normal seeds of some plants may store ten times the total molybdenum needs of the plant to be grown from the seed" and that to show molybdenum essentiality the use of specially grown seeds with low molybdenum content were required.

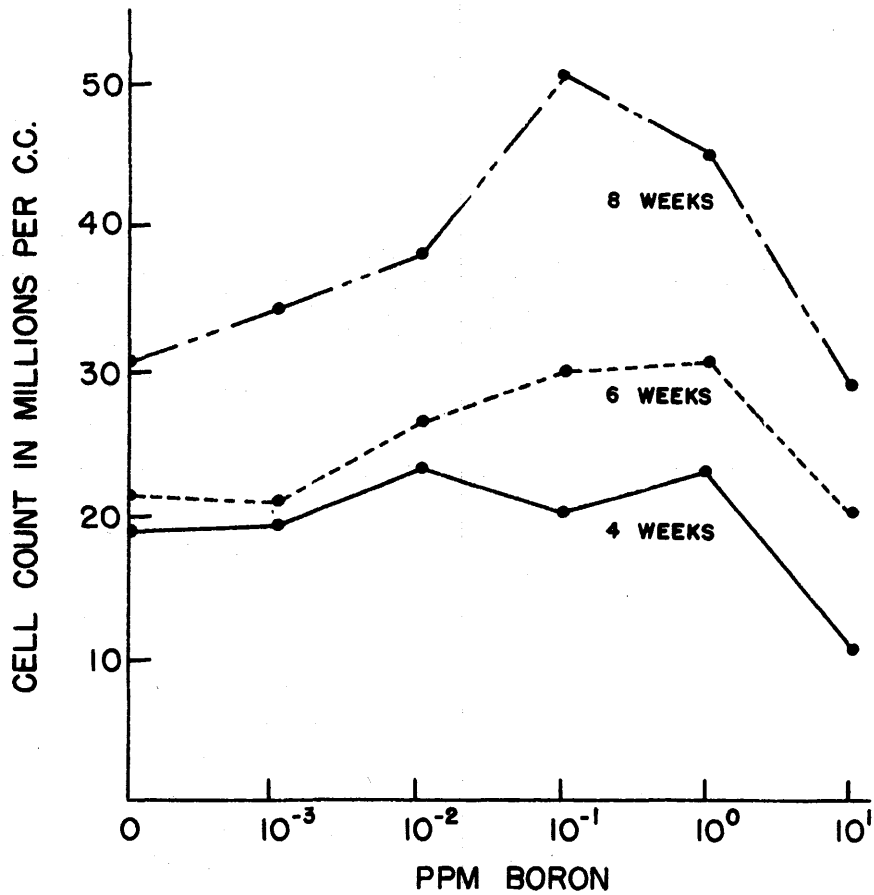

FIGURE 6. Growth of *Nostoc muscorum* at successively higher levels of boron in the medium.

Iron deficiency symptoms (fig. 1) were demonstrated easily even without "specpure" salts and without a subculture inoculum. The data show that the minimum requirement of iron for maximum growth in Chu No. 10 medium was about 0.08 p.p.m. iron, which is only 30 percent of the amount recommended for modified Chu No. 10 medium. C.P. grade ferric chloride was contaminated with manganese so that no manganese deficiency could be demonstrated until a spectroscopically manganese-free source of iron was used. Manganese essentiality is indicated in figures 2 and 5. There were striking differences in densities and cell counts between a culture with "0" manganese and a culture with 0.001 p.p.m.

manganese. The molybdenum requirement for the growth of *Nostoc muscorum* is shown in figures 4 and 7. Boron essentiality is indicated in figures 3 and 6. Cultures with 0.1 p.p.m., boron showed significantly higher cell counts than cultures with "0" boron, which developed extreme chlorosis. Although there was less extreme chlorosis with 0.001 p.p.m. boron than with "0" boron, there was very little difference in the cell counts. Boron-deficient cultures became entirely white

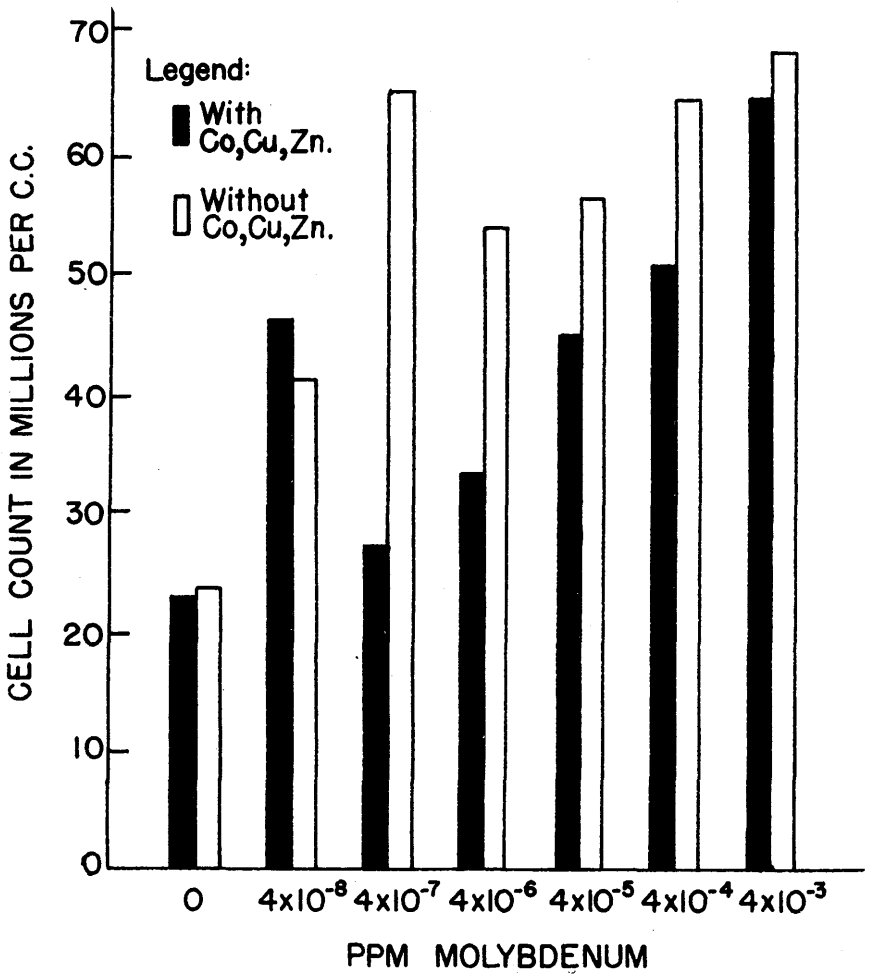

FIGURE 7. Growth of *Nostoc muscorum* at successively higher low levels of molybdenum, comparing growth in the presence of cobalt, copper, and zinc (0.01 p.p.m. of each) with growth in the absence of cobalt, copper and zinc.

as did iron-deficient cultures. Some iron-deficient cultures started out with a blue color changing to white with age. Molybdenum deficiency was characterized by a yellowish-green color, and blue was the characteristic color of manganese-deficient cultures. The term "blue chlorosis" may be used to describe manganese-deficient *Nostoc* cultures.

A molybdenum-copper relationship.—In the presence of cobalt, copper, and zinc (0.01 p.p.m. of each) salts the cell counts of the cultures at various low levels of

molybdenum were consistently lower than similar cultures without cobalt, copper, and zinc (fig. 7). The only exception was at 4 x 10^{-8} p.p.m. molybdenum. Subsequent cultures with separate additions of cobalt, copper and zinc have shown the effect to be due largely to copper. Molybdenum and copper have previously been found to be reciprocally antagonistic in animal metabolism (Cunningham, 1950). Copper probably competes with molybdenum for a position in the structure of a molybdenum containing enzyme, the active enzyme molecules being the ones containing molybdenum.

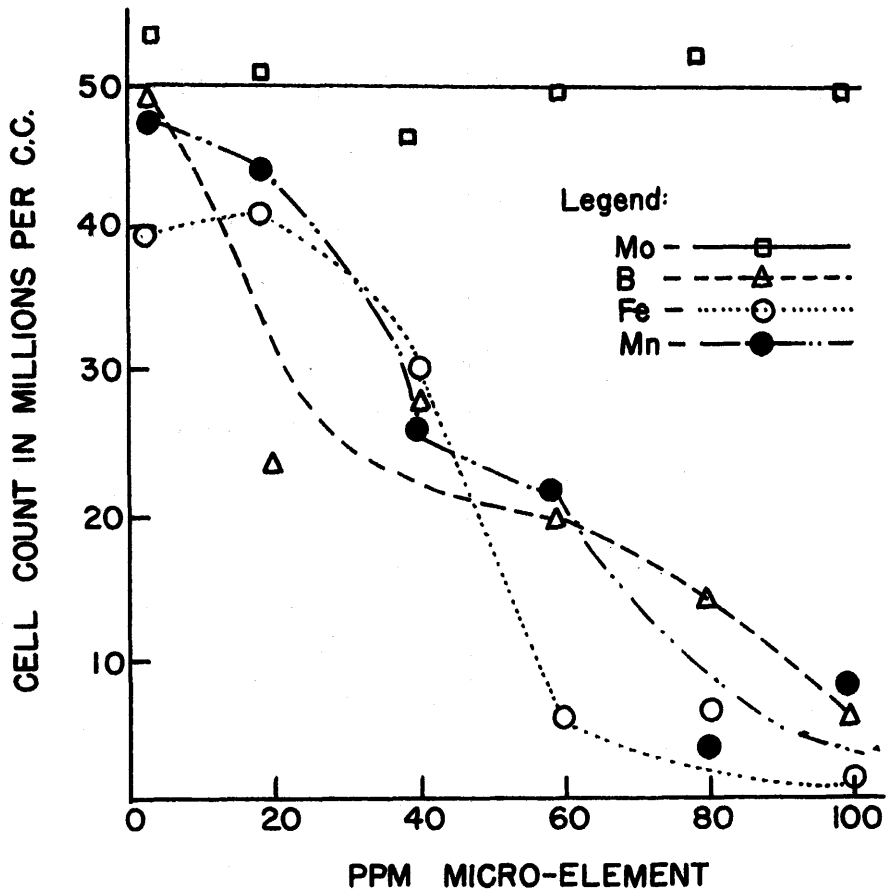

FIGURE 8. Toxic effect on the growth of *Nostoc muscorum* produced by higher than optimal concentrations of molybdenum, boron, iron and manganese, respectively.

Toxicity experiments.—Table 3 and figure 8 present data on the toxicity of the four micronutrient elements. The addition of large amounts of iron and manganese salts caused a shift in the pH, which was adjusted with 0.1 N NaOH to between pH 8.5 and 9.0. Iron showed no toxicity up to 20 p.p.m. as was also the case for manganese. Boron and molybdenum toxicity began at above 1 p.p.m. boron and above 100 p.p.m. molybdenum, respectively. There was almost no growth of *Nostoc muscorum* at 60 p.p.m. iron, 80 p.p.m. manganese, 100 p.p.m. boron, or 500 p.p.m. molybdenum. These were approximate minimum concentrations which inhibited growth almost completely.

SUMMARY

Cultures of *Nostoc muscorum* were grown in Chu No. 10 medium which was prepared with spectroscopically pure compounds. The cultures were illuminated continuously with fluorescent light of 100 f.c. intensity at a temperature of approximately 30° C. The cultures were shaken by hand usually once per day and were neither bubbled with air nor with CO_2 mixed with air. The micronutrient elements found to be essential to the growth of *Nostoc muscorum* were iron, manganese, boron, and molybdenum. Copper was found to interfere with the utilization of molybdenum. Iron showed no toxicity up to 20 p.p.m. as was also the case for manganese. Boron and molybdenum toxicity began at above 1 p.p.m. boron and above 100 p.p.m. molybdenum, respectively. Approximately minimum concentrations which inhibited growth almost completely were 60 p.p.m. iron, 80 p.p.m. manganese, 100 p.p.m. boron or 500 p.p.m. molybdenum.

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